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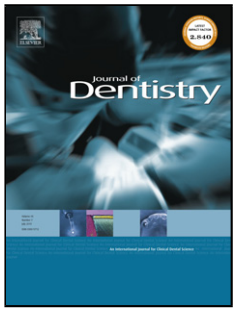

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An in-situ pilot study to investigate the native clinical resistance of enamel to erosion.

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Keywords

Enamel, tooth wear, profilometry, surface roughness, saliva, pellicle.

Abstract

Objectives: To investigate the differences in susceptibility of the surface of native and polished enamel to dietary erosion using an in-situ model.

Methods: Thirty healthy volunteers (n=10 per group) wore mandibular appliances containing 2 native and 2 polished enamel samples for 30 minutes after which, the samples were exposed to either an ex vivo or in vivo immersion in orange juice for 5, 10 or 15 minutes and the cycle repeated twice with an hour's interval between them. Samples were scanned with a non-contacting laser profilometer and surface roughness was extracted from the data, together with step height and microhardness change on the polished enamel samples.

Results: All volunteers completed the study. For native enamel there were no statistical difference between baseline roughness values versus post erosion. Polished enamel significantly increased mean (SD) Sa roughness from baseline for each group resulting in roughness change of 0.04 (0.03), 0.06 (0.04), 0.04 (0.03), 0.06 (0.03), 0.08 (0.05) and 0.09 (0.05) μm respectively. With statistical differences between roughness change 45 minutes in vivo versus 45 minutes ex vivo ($p<0.05$). Microhardness significantly decreased for each polished group, with statistical differences in hardness change between 30 minutes in vivo versus 30 minutes ex vivo ($p<0.05$), 45 minutes in vivo versus 30 minutes ex vivo ($p<0.01$), 45 minutes in vivo versus 45 minutes ex vivo ($p<0.01$).

Conclusions: The native resistance to erosion provided clinically is a combination of the ultrastructure of outer enamel, protection from the salivary pellicle and the overall effects of the oral environment.

ClinicalTrials.gov Identifier: NCT03178968

Clinical significance: This study demonstrates that outer enamel is innately more resistant to erosion which is clinically relevant as once there has been structural breakdown at this level the effects of erosive wear will be accelerated.

Introduction

The first sign of erosive tooth wear is described as early surface texture loss. There have been recent developments this surface change using surface roughness parameters [1]. Surface roughness measurements have been specifically advocated for erosion studies investigating early erosion changes without tissue loss [2]. The origins of surface roughness measurements come from nanometrology and are based upon the principle that every surface is combined of three component forms; (profile), waviness and roughness (which combined are texture). The form is described as the underlying shape, whereas waviness and roughness are deviations from the shape. In particular roughness is the minute wavelengths that give an indication of the nature of a substance [3,4]. When quantifying changes in enamel following erosive wear using surface roughness measurements it is the activity at the level of an enamel prism that is being measured, which has been postulated to be relevant for quantifying initial erosion [2]. Different parameters are used to quantify surface roughness including amplitude. Amplitude parameters calculate roughness as height deviations from the form. The parameter used in this study was Sa roughness, which is a 3 dimensional measure which represents the mean roughness of a studied surface [5].

Most erosion studies use enamel samples that have been polished flat, a process which removes the outer layer of enamel and alters the overall form and creates a surface that is easier and more reproducible to measure [6,7]. The effects of erosion have been recently investigated in vitro using both polished and native enamel samples (where the outer surface has been left intact) [8–10]. We reported data from in vitro studies using surface roughness changes on polished enamel and native enamel over different locations, and identified that five discrete measurements from the centre of native enamel were representative of the overall surface of the sample [9]. We also identified that whilst polished enamel becomes rougher, native enamel becomes smoother after 45 minutes' immersion in orange juice [8,9]. However, polished enamel exhibited statistically significant changes in surface roughness after only 15 minutes' immersion in orange juice whereas it took 45 minutes for

native enamel to show quantifiable change. This suggests that as well as behaving differently to polished enamel, native enamel is innately resistant to erosion.

The native enamel surface provides a more clinically relevant substrate, however, to be truly clinically representative the effects of the total native defences to erosion must be investigated. In vitro studies can overestimate erosive changes by approximately 10 fold [11]. Currently there are no validated methods to measure surface roughness in-vivo. Therefore, the optimum method to simulate the clinical environment is an in-situ study with in-vivo erosion, which was used in this study. The aim of this study was to investigate the differences in susceptibility of native and polished enamel to dietary erosion using an in-situ model and compare the influence of native biological defences to erosion. The null hypothesis was: there is no change to the enamel surface following immersion in orange juice.

Methods

Experimental design

This was a single-blind, randomised intervention study involving 30 healthy volunteers (10 volunteers per study group) who met the inclusion/ exclusion criteria, to measure surface changes of native enamel and polished enamel *in-situ* following an orange juice acid challenge. Ethical approval for the study was granted by the Stanmore Health Research Authority REC ref 15/LO/0417, and the study was conducted using the guidelines for Good Clinical Practice. The inclusion criteria stipulated mild erosive tooth wear maximum score of 2 in each sextant and cumulative score no more than 8, aged 18 years and over, willing to participate, not enrolled in any other research, possessing more than 20 anterior and posterior teeth, no active carious lesions and a maximum BPE score of 2 in one sextant (no periodontal disease). The exclusion criteria stipulated pregnancy or breast feeding, medical history likely to impact on attendance or mobility, insulin dependent diabetes, saliva diagnoses (xerostomia), lower orthodontic appliances, dentine hypersensitivity, defective restoration of the occlusal or incisal surfaces of upper anterior teeth and first molars and any condition that precluded consumption of 300 mL of orange juice a day for 5 consecutive days. Following recruitment into the study, lower

impressions were recorded in alginate, using stock trays, to fabricate custom-made lower soft orthodontic appliances. The appliances were designed to accommodate four enamel samples (one polished and one native on each side) and positioned buccally in the premolar/molar region. The volunteers underwent a 5 day wash out period during which they used a non-fluoridated toothpaste (Kingfisher, Norwich, UK) and standard manual toothbrush. They were also asked to refrain from eating or drinking for two hours prior to the start of the study appointment.

Extracted human molars without visible signs of caries or tooth wear were stored in sodium hypochlorite for a minimum of three days [9]. The roots were removed and the crowns sectioned using a circular diamond saw (XL 12205, Benetec Ltd., London, UK) to produce 120 (4 x 4 mm) buccal enamel sections. These enamel sections from the buccal surfaces of teeth were randomly allocated to produce 60 native and 60 polished (flattened) samples. Both groups were embedded in bisacryl composite (Protemp4 3M ESPE, Germany) using custom made mould trays ensuring the outer curved surfaces remained untouched; and were cleaned using a soft toothbrush and non-fluoridated toothpaste (Kingfisher, Norwich, UK) and wiped with ethanol. The polished samples, were fully embedded in bisacryl composite, placed in a water-cooled rotating polishing machine (LaboForce 100, Struers, ApS, Ballerup, Denmark) and polished flat using a series of Silica Carbide Grits (Versocit, Struers A/S, Copenhagen, Denmark) to produce 60 optically flat samples with a flatness tolerance within 0.4 µm [12]. Following preparation all the samples (native and polished) were ultrasonicated in deionised water for 15 minutes and immersed in sodium hypochlorite prior to baseline measurements being recorded. For the polished samples, PVC tape was applied over the enamel to create a window of exposed enamel (1 mm) with a reference area of enamel either side (each 1 mm) and used for step height measurement.

Three erosion times were investigated at 15, 30, or 45 minutes, which were achieved using a 3-cycle model. The volunteers were randomly allocated into these groups using statistical software (GraphPad). Ex vivo and in vivo erosion was carried for each participant. For the ex vivo erosion one

native and one polished sample were removed from the splint and immersed in 20 mL of orange juice and agitated at 62 rpm (Stuart Scientific, Mini Orbital Shaker S05, Bibby) for either 5, 10, or 15 minutes. Following which they were reinserted into the splint and worn during the rest periods. During the ex vivo erosion the participant rinsed with orange juice for the same time duration. The process for in-vivo and ex-vivo was repeated twice with an hour's rest between giving 3 cycles of erosion thereby totally 15, 30 or 45 minutes' erosion, a flow chart of the study schedule is shown in Figure 1. After the 3rd and final erosion cycle the volunteers were given a desensitising toothpaste (Sensodyne Repair & Protect, GSK, Weybridge, UK).

After removal of the sample from the splint samples were ultrasonicated in sodium hypochlorite for 30 minutes [13].

Surface (Sa) roughness was quantified by selecting five representative areas (each 0.04 mm²) from the centre area of native and polished samples [9]. These areas were scanned using a 4 µm scanning interval at baseline and after erosion with a red laser (2 µm spot size) non-contact confocal profilometer (Xyris 2000, TaiCaan, Southampton, UK) following a previously published protocol. The resulting scan images were analysed for Sa roughness (MountainsMap, DigitalSurf, France) by levelling the samples, applying a 25 µm Gaussian filter, [9] and roughness change calculated by subtracting the mean eroded roughness value from baseline. The % roughness change was calculated by dividing the absolute roughness change from the baseline value multiplied by 100. To measure step height loss a second larger scan (approximately 3 mm by 3mm) with a scanning interval of 10 µm was carried out for each polished enamel sample after erosion. The resulting scan images were analysed using BODDIES analysis software (Southampton, UK). Three profiles were extracted and the vertical drop measured from the midpoint of the eroded zone to each reference area in accordance to ISO 5436 standards. This provided a total of six measurements per sample which were averaged.

Microhardness testing was carried out at baseline and after erosion for polished enamel samples using a Knoop Microhardness Indenter (Duramin-5, Struers Ltd, Rotherham, UK). Each polished sample had

three indentations taken 100 µm apart using a press load of 981.2 mN and a press time of 10 seconds from a reference area at baseline and the eroded zone after erosion. Knoop microhardness was calculated using the Duramin software (Struers, Catcliffe, UK) and the Knoop microhardness change (KHC) calculated by subtracting the average of the worn and baseline reference areas for each polished sample.

Statistical analysis

This was a pilot study, therefore no formal powering could be carried out, however it was estimated based upon previous in vitro studies which compared the correlation of surface roughness with other markers of erosion (enamel microhardness), which suggested a total sample of 20 to achieve the power of 80% using Gpower version 3.1.5.

SPSS version 22 was used to analyse the data. Normality was checked using Histogram plots and Shapiro Wilk tests. The data were normally distributed, with any data not originally normally distributed Log transformed. ANOVAs with post hoc Tukey tests to determine inter and intra group significance. Significance was set at $P<0.05$.

Results

A total of 40 potential volunteers were assessed and from which 30 volunteers fulfilled the inclusion and exclusion criteria and completed the study. The participants had a mean age of 29.6 years (range 20 to 54 years) and female to male ratio of 2:1. Two volunteers reported sensitivity the day after their study visit; one had 45 minutes' exposure and the other 30 minute but application of a fluoride varnish Duraphat®, Colgate®, Colgate-Palmolive, Germany) provided relief within 24 hours together with the addition of an application of self-bond (Scotchbond, 3M, USA).

The roughness data is shown in Table 1. For native enamel there were no statistical changes in Sa roughness after the interventions. For polished enamel there were statistical differences between roughness change after 45 minutes in-vivo versus 45 minutes ex-vivo ($p<0.05$).

Step height and microhardness were not measurable for the native samples. The mean (SD) Knoop microhardness change for groups 15, 30 and 45 minutes in-vivo of polished enamel were 159.6 (66.1), 190.8 (82.1) and 168.2 (78.2) and for ex vivo were 190.9 (59), 241.9 (27.9) and 246.9 (36.9) respectively. There were statistical differences in KHC between 30 minutes ex-vivo versus 30 minutes in-vivo ($p<0.05$), 30 minutes ex-vivo versus 45 minutes in-vivo ($p<0.01$) and 45 minutes in-vivo versus 45 minutes ex-vivo ($p<0.01$). The mean (SD) step height for groups 15, 30 and 45 minutes in-vivo of polished enamel were 2.16 (2.50) µm, 2.62 (2.03) µm and 2.43 (2.62) µm respectively and for ex-vivo were 2.26 (1.23), 2.42 (1.07) µm and 3.60 (2.68) µm. There were no statistical differences between these values ($p>0.05$). KHC and step height for each group are shown in Table 2.

Discussion

There was no statistical difference in mean (SD) Sa roughness of native enamel before erosion compared to after erosion, even after 45 minutes of immersion in orange juice ex-vivo. Whereas polished enamel became significantly rougher after only 15 minutes. Which supports previous suggestions that native enamel is more resistant to erosion [8,14,15]. A recent study also identified significantly less calcium release during erosion cycling for native enamel compared to polished enamel, again suggesting innate resistance of native enamel [16]. The significant differences between 45 minutes of erosion in-vivo versus ex-vivo suggests that the overall oral environment offers further resistance to erosion compared to only presence of the applied pellicle.

Despite the data showing no statistical change from baseline for native enamel and polished enamel becoming significantly rougher, the values for absolute roughness change were very similar for native and polished enamel with all roughness change being less than 0.10 µm. Therefore, it could be suggested that when absolute change is considered the polished and native samples behave similarly. However, we would suggest that to interpret the effect of roughness change the baseline values must be considered. Native enamel had baseline roughness values around six times greater than polished enamel, therefore whilst the value of the change may be similar its physiological effect is reduced.

This was evident when % roughness change was considered. The same absolute value of roughness change (0.04 µm) resulted in a percentage change of 225% for polished enamel but only 20 % for native enamel. Therefore, the impact of absolute change is very different. During the filtering process the form of the measured sample is removed and surface roughness is calculated from the remaining wavelengths which result from the structural components of the substance [4]. The same filter was used for both substrates based upon 5 times the size of an enamel prism. However, whilst the basic chemistry of polished enamel and native enamel are the same, hydroxyapatite crystallites which form hexagonal prisms (3-6 µm in diameter) separated by organic proteins [17–19], there are different structural components. Native enamel contains an outer layer, which was removed during the polishing process. This layer contains areas devoid of prisms known as aprismatic enamel and In these areas the prism junctions and orientation are often highly complex and no clear prism structure is identifiable [20]. Previous studies which have investigated polished and native enamel, measuring Sa roughness and conducting qualitative assessment with SEM, have associated the higher roughness values for native enamel with its complex structure and textural features [9,21]. Therefore, we would suggest that the absolute values of roughness change can be misleading and the impact these have on the different substrates must also be considered. This is clinically relevant as once breakdown of the outer enamel has occurred the underlying enamel structure would be more similar to laboratory polished enamel. Therefore the statistical effects of roughness change on the polished samples could have clinical relevance for severe erosion. Surface roughness measurements can give an indication of change within areas of structural breakdown as shown by the polished enamel samples becoming significantly rougher in the eroded zone where step height loss was also occurring. Previous in vitro studies have identified that significant roughness change occurred in areas of structural breakdown, identified through SEM imaging [8,9].

The basics of the in-situ model used in this study were previously tested in vitro. We reported significant changes in surface roughness of native enamel after 45 minutes' immersion with the same

orange juice and agitation used in this study, which followed trends identified in other studies [8–10,22]. However, this was not the case when an in-situ model was applied. In this in-situ study there were no trends in whether native enamel became smoother or rougher, and within each of the 6 groups (n=10 samples per group) individual native enamel samples had a variable response to erosion with some becoming rougher or smoother. This may be due to natural variation of native enamel, the samples were prepared from different mouths which could influence the difference in susceptibility of the individual samples. However, the previous in vitro studies which did identify clear trends would dispute this. Therefore, another influence could be the different oral environments of the participants. Uhlen et al. 2016., [23] investigated the susceptibility of native enamel to erosion at a profile level. Their in-situ model simulated intrinsic induced erosion and used step height as the outcome measure. They reported significant differences in susceptibility to erosion between donors and identified significant differences in lesion depths amongst the in-situ hosts. The authors suggested that the conditions of the oral environment were more influential in protection from erosion than the innate characteristics of the native teeth [23]. Therefore, whilst an in vitro model attempts to suppress variability it does not represent the clinical situation and the variation in roughness change of native enamel observed in our study may be indicative of the in-vivo resistance to erosion.

Within the polished enamel groups the samples were less variable and all became rougher after erosion, even after 15 minutes' erosion in-vivo. This suggests that following clinical breakdown of outer enamel erosive wear would continue at an accelerated rate. Despite native enamel being more clinically representative polished enamel samples can be informative when investigating mode of action, such as the remineralising effects of saliva. Step height measurements were not possible from native enamel as the angular tolerance of the red laser sensor resulted in data drop out over the extended curvature of the full size of samples and the 0.004 mm^2 areas that were imaged did not provide identifiable features required for superimposition techniques to be used. Microhardness measurements were not possible due to the natural curvature of the native enamel samples.

Therefore, polished enamel samples were necessary for this study to investigate the innate resistance to erosion offered by the overall oral environment. The ex-vivo acid challenges in this study allowed investigation into the protection offered by pellicle formation. The salivary pellicle is a thin biofilm that contains proteins, glycoproteins, mucins and enzymes that coats the surfaces of teeth and is believed to have a protective effect from its carbonhydrase species of proteins [13,24]. To allow pellicle formation the volunteers wore the intra oral appliance for 30 minutes prior to the first acid challenge and for 1 hour between each acid challenge. The roughness, step height and microhardness data of polished enamel samples demonstrated increased roughness change, step height loss and hardness change with increased ex-vivo acid exposure. This supports previous suggestion that the protective effect of the pellicle only lasts 10 minutes [25]. The mean (SD) step height measurements of polished enamel after immersion in orange juice have similarities to other studies. Moazzez et al. 2014 [26] identified a step height loss of 1.34 (0.66) µm on pellicle coated polished bovine enamel samples following *ex-vivo* immersion in Citric Acid pH 3.2 for 10 minutes.

In-vivo, saliva offers resistance to erosion from pellicle formation, buffering ability to neutralise acids, remineralisation and the physiological effect of washing away debris [13,24,27,28]. Therefore, it provides resistance using a complex network of biological factors and the in-vivo immersion data reflected this. Trends in roughness change, step height loss and hardness change of polished enamel increased between 15 and 30 minutes' erosion then decreased between 30 and 45 minutes. It is unclear why there was reduction in the outcome measures of the erosion between 30 and 45 minutes. An early replica study of erosive lesions in-vivo identified different morphologies of erosive wear, ranging from irregular pitted surfaces to smooth lesions [29]. However, it was not possible attribute the morphology to lesion activity. It has been suggested that erosive wear is not a linear consistent process but consists of bursts of activity and quiescent periods [30]. Studies which have investigated demineralising-remineralising cycles using polished enamel samples have identified that polished becomes rougher after demineralising in acid then smoother (although not as smooth as baseline)

following remineralising [31]. Furthermore natural saliva has been shown to exhibit a remineralising effect similar to fluoride [32]. It is likely that within the acid challenges in this study there was a combination of roughness increases and decreases. This is indicated by statistical differences in roughness change and hardness change between 45 minutes' erosion ex-vivo versus 45 minutes' erosion in-vivo. Overall identifying that saliva protection is combined from the pellicle layer and full physiological function in-vivo.

Thereby, solely relying on in-vitro and ex-vivo erosion models are not indicative of the clinical scenario and overestimate erosive wear. We would also suggest that whilst native enamel is a substrate upon which it is difficult to quantify early structural changes, it is clinically representative and more research investigating native enamel is required. However, we acknowledge that for some studies, particularly those investigating mode of action, polished enamel may remain the substrate of choice until further advancements in metrological techniques are made.

Conclusions

The natural resistance to erosion provided clinically is a combination of the ultrastructure of outer enamel, the salivary pellicle and the overall oral environment. All of these must be considered to produce a clinically relevant study model.

Figures and Tables

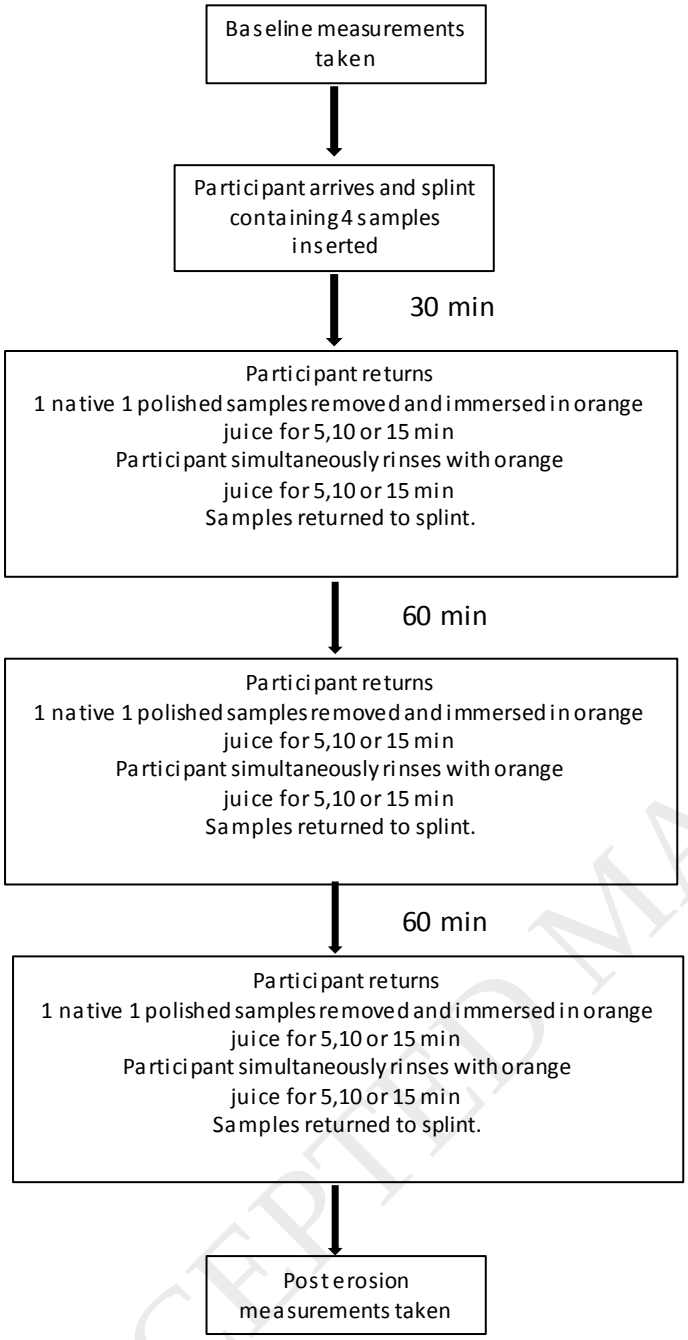

Figure 1: Flow chart of study visit.

Table 1: Mean (SD) Sa roughness of native and polished enamel before and after *in-vivo* and *ex-vivo* acid challenges 15min, 30min and 45min and Mean (SD) Sa roughness change. ^{ns}=p>0.05, *= p<0.05 before vs after, ^{1}= statistical significance between roughness change 45 minutes in-vivo vs 45 minutes ex-vivo p<0.05.

	Mean (SD) Sa Before (µm)	Mean (SD) Sa After (µm)	Mean (SD) absolute Sa change (µm)	Mean % Sa change (%)
Native enamel				

15 min	**In-vivo**	0.26 (0.12)	0.33 (0.10) [ns]	0.07 (0.01)	27
	Ex-vivo	0.31 (0.14)	0.23 (0.08) [ns]	-0.07 (0.05)	-23
30 min	**In-vivo**	0.25 (0.14)	0.18 (0.08) [ns]	-0.07 (0.06)	-28
	Ex-vivo	0.27 (0.10)	0.32 (0.13) [ns]	0.05 (0.03)	19
45 min	**In-vivo**	0.20 (0.07)	0.25 (0.10) [ns]	0.05 (0.03)	25
	Ex-vivo	0.20 (0.08)	0.24 (0.09) [ns]	0.04 (0.01)	20
Polished Enamel					
15 min	**In-vivo**	0.04 (0.02)	0.08 (0.04) *	0.04 (0.03)	100
	Ex-vivo	0.04 (0.01)	0.09 (0.03) *	0.06 (0.03)	150
30 min	**In-vivo**	0.04 (0.01)	0.10 (0.04) *	0.06 (0.04)	225
	Ex-vivo	0.04 (0.01)	0.12 (0.04) *	0.08 (0.05)	200
45 min	**In-vivo**	0.04 (0.01)	0.07 (0.03) *	0.04 (0.03) [1]	100
	Ex-vivo	0.04 (0.01)	0.13 (0.04) *	0.09 (0.05) [1]	225

Table 2: Mean (SD) Sa roughness change, Microhardness change and Step Height for polished enamel samples after *in-vivo* and *ex-vivo* acid challenges 15min, 30min and 45min. [a]= statistical significance 30 min in-vivo vs 30 min ex-vivo p<0.05, [b]= 45 min in-vivo vs 45 min ex-vivo p<0.01, [c]=45 min in-vivo vs 30 min ex-vivo p<0.01

	Mean (SD) Microhardness Change (KHN)		**Mean (SD) Step height (µm)**	
	In-vivo	**Ex-vivo**	**In-vivo**	**Ex-vivo**
15 min	159.6 (66.1)	190.9 (59.0)	2.16 (2.50)	2.26 (1.23)
30 min	190.8 (82.1) [a/c]	241.9 (27.9) [a/b]	2.62 (2.03)	2.42 (1.07)
45 min	168.2 (78.2) [b/c]	246.9 (36.9) [b]	2.43 (2.62)	3.60 (2.68)

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Figure 2: Flow chart of study visit.